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Archives of Biochemistry and Biophysics 438 (2005) 182–194
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Gene profiling the effects of calcium deficiency versus 1,25-dihydroxyvitamin D induced hypercalcemia in rat kidney cortex ☆

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Received 9 March 2005, and in revised form 20 April 2005
Available online 10 May 2005

Abstract

Determinants involved in the activation and repression of 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) synthesis in renal cortex by changes in extracellular Ca were studied. Cortical kidney RNA isolated from hypocalcemic (LC) rats generated by a low Ca diet, and hypercalcemic (HC) rats generated by a normal Ca diet and two sequential 1 μg doses of $1,25(OH)_2D_3$. Among the genes up-regulated were 1α-OHase (4.6-fold) in the LC group and high differential gene expression of VDR (4.0-fold) and 24-OHase (10.4-fold) in the HC group. Moreover, the exposure of renal cortex to LC versus HC conditions revealed a high differential expression of a PKA-dominated pathway involving CBP interacting protein, GATA-1 and CREB transcription factors in the LC model. In the HC model, elevated renal cortex gene expression of several growth factors, peptide receptors, and intracellular signaling molecules depicts a role for CaSR activation and receptor tyrosine kinase signaling in $1,25(OH)_2D_3$-mediated gene activation and repression of 1α-OHase.

Keywords: Vitamin D receptor; 1α-OHase; 24-OHase; Calcium; Microarray; PTH

The vitamin D endocrine system is principally regulated in the kidney proximal tubule by coordinated responses to ionized extracellular calcium (Ca^{2+}) [1,2]. Increased blood Ca^{2+} is detected by the extracellular Ca^{2+} sensing receptor (CaSR) in the parathyroid gland [3], resulting in the reduced secretion of parathyroid hormone (PTH), whereas, low blood Ca^{2+} elevates PTH release. PTH has specialized functions in the kidney proximal tubule which include stimulation of renal 25-hydroxy-1α-hydroxylase (1α-OHase) and targeting the stability of the 25-hydroxy-24-hydroxylase (24-OHase) mRNA transcript [4]. Conversely, either high blood Ca^{2+} or an abnormal increase in serum 1,25-dihyroxyvitamin D ($1,25(OH)_2D$) can block renal proximal 1α-OHase gene expression and activity in vivo [5,6].

In principle, $1,25(OH)_2D$ regulates the renal 1α-OHase by classical negative feedback [7], but the mechanism of this repressive effect is not understood. The involvement of the $1,25(OH)_2D$ receptor (VDR) is thought to be one factor necessary for the repression of 1α-OHase by $1,25(OH)_2D$ [8]. The role of VDR-mediated repression of 1α-OHase has been addressed by a series of direct and indirect studies previously reviewed [9,10]. Exogenous treatment of $1,25(OH)_2D$ to rats fed a normal Ca diet results in potent down-regulation of both 1α-OHase gene expression and enzymatic activity [11,12]. In contrast, endogenous production of $1,25(OH)_2D$, induced by dietary Ca deficiency, can reach blood levels that are four to five times above nor-

☆ This work was supported by grants from the A.D. Williams Foundation (Virginia Commonwealth University Health System), the Virginia Jeffress Foundation, and a USDA, CRA 58-3625-2-155.
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doi:10.1016/j.abb.2005.04.016

mal without any apparent repression on 1α-OHase [5,11]. One possible reason for this is the decrease in renal VDR, which drops to 20% of control following prolonged Ca deficiency. Also, VDR targeted mutation greatly elevates 1α-OHase gene expression [8,13]. Analysis of the 1α-OHase promoter activity demonstrated that $1,25(OH)_2D_3$ treatment in vitro, with or without PTH co-treatment, does not decrease 1α-OHase promoter activation [14]. This is not surprising given that a canonical VDR response element (VDRE) has yet to be identified in the 1α-OHase promoter [15]. Collectively, these studies indicate an elaborate but indirect mechanism for VDR-mediated repression of 1α-OHase that is proposed to involve sensitivity to extracellular Ca [16]. PTH, on the other hand, induces 1α-OHase by a cAMP led mechanism in the proximal tubule [6,14,17], and also influences renal proximal 24-OHase transcript levels as well as promoting VDR down-regulation in proximal but not distal nephron segments [18–23]. The molecular mechanism and determinants by which PTH induces 1α-OHase gene expression are also unclear.

The goal of this study was to better define the intermediates involved in regulating proximal kidney cell activation and repression of $1,25(OH)_2D_3$ synthesis. To do this, GeneChips for oligonucleotide microarray analysis were utilized between two conditionally opposing models of Ca^{2+} metabolism characterized by hypocalcemia on one end and hypercalcemia on the other. The hypercalcemia model was a result of acute $1,25(OH)_2D_3$ treatment at 16 and 4 h before sacrifice, resulting in gene regulation that was affected by $1,25(OH)_2D_3$ and the attendant rise in blood Ca^{2+}. This gene profiling approach allowed for mapping of the vitamin D endocrine system strictly based upon in vivo responses to coordinated Ca^{2+}, PTH, and $1,25(OH)_2D$ actions, and also provided a comprehensive search for new determinants in renal vitamin D metabolism and proximal tubular phosphate handling.

Materials and methods

Animals and diets

All animal handling, treatments, and medications were IACUC approved at the National Animal Disease Center, Ames, IA, before execution of the study. The diets used in the study are depicted in Table 1. Weanling rats (Harland Spague–Dawley, Madison, WI) were fed diets containing 0.02% calcium (LC) or 0.47% calcium (TekLad, Madison, WI) for 3 weeks. The phosphate level in each diet was 0.30%. Some of the LC and NC rats were given daily oral doses of different levels of vitamin D_3, 0 (DD), or 4 μg D_3 per day for a normal growth control diet (Cntrl diet). Also, one group of the LC/DD and Cntrl diet rats, respectively, was given sequential injections of 1 μg of $1α,25(OH)_2D_3$ (EMD-Calbiochem, San Diego, CA) at 16 and 4 h before sacrifice (1,25-trt) to stimulate increased blood Ca, LC/DD/$1,25D_3$ and hypercalcemia (HC), respectively [24]. Blood calcium measurements were performed using atomic absorption spectrophotometry.

Isolation of RNA and DNA removal

Total RNA was isolated by tissue extraction from 100 mg of kidney cortex or 1 cm of upper intestinal duodenum using Trizol (Invitrogen). Kidney total RNA from LC and HC rats was purified by RNeasy oligo-deoxythymidine column chromatography (Qiagen, Valencia, CA). RNA samples were further purified using GenHunter's MessageClean Kit, which removed any DNA contaminations from RNA. The purified RNA was used for GeneChip microarray and in real-time RT-PCR analysis.

Microarray

Microarray was conducted at NARF (Nucleaic Acid Research Facilities of Virginia Commonwealth University). RNA samples were hybridized on the Rat Genome U34A GeneChip Set, which probed for 7000 known rat gene sequences plus 1000 expressed sequence tag (EST) gene cluster sequences. Pooled RNA samples from 3 rats per diet were labeled using the Enzo BioArray High-Yield mRNA Transcript Labeling Kit that is required for use with GeneChip eukaryotic expression arrays. The procedure involved transcription-based amplification and biotin labeling of RNA for hybridization to the GeneChip expression arrays. The GeneChips were hybridized by using the Eukaryotic Hybridization Control Kit purchased from Affymetrix (Santa Clara, CA). Pre-mixed biotin-labeled *bioB*, *bioC*, *bioD*, and *cre*

Table 1
Diets used in study: renal cortical RNA samples from LC and HC (bold) were compared using GeneChip microarray analysis

Component	Cntrl diet	LC/DD	LC/DD/1,25	LC	HC
Diet Ca	0.47%	0.02%	0.02%	**0.02%**	**0.47%**
Diet P	0.30%	0.30%	0.30%	**0.30%**	**0.30%**
Vitamin D_3	4 μg per day	0	0	**4 μg per day**	**4 μg per day**
$1,25\text{-}D_3$ trt	0	0	2 doses[a]	**0**	**2 doses**[a]

[a] One microgram of $1,25(OH)_2D_3$ given subcutaneously at 16 and 4 h prior to sacrifice.

eukaryotic hybridization controls were added directly to the hybridization cocktail in staggered concentrations. These spiked controls facilitated monitoring of the hybridization process for troubleshooting. In addition, this kit also contained control Oligo B2, used to provide alignment signals for the Affymetrix Microarray Suite software. Once the GeneChips were hybridized the data were read by a GeneArray Scanner, from Affymetrix. The scanned data were analyzed using the Affymetrix Microarray Suite software package. To compare array data between treatments the average of the fold differences in gene expression as determined by fluorescent signal intensities of all probes on each array was used.

Quantitative RT-PCR

The experiments were performed in the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan One Step PCR Master Mix Reagents Kit (P/N: 4309169). All the samples were tested in triplicate under the conditions recommended by the fabricant. The cycling conditions were: 48 °C/30 min; 95 °C/10 min; 40 cycles of 95 °C/15 s and 60 °C/1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98–1.0). The probes and primers were designed using the Primer Express 2.0 version. The probes were labeled at the 5′ end with FAM (6-carboxyfluoresceine) and at the 3′ end with TAMRA (6-carboxytetramethylrhodamine). L19 and Ribosomal RNA (18s rRNA) from the Pre-developed TaqMan Assay Reagents (P/N: 4310893E) was used as endogenous control. Specific gene expression analysis was conducted using TaqMan probes to several gene products of interest (24-OHase, 1α-OHase, VDR, CaSR, GATA-1, and CREB).

Renal proximal cells and transfection

Cell cultures of renal epithelial proximal tubule cells (HKC-8, a gift from Lorraine Raucusen, Johns Hopkins University) were grown in 50% Dulbecco's modified Eagles medium and 50% Ham's F12 medium (DMEM:F12). The medium contained 10% by volume of fetal bovine serum and penicillin streptomycin as antibiotics. The cells were divided 1:3 to subculture and fed fresh medium once a week. A plasmid expression construct containing cDNA of GATA-1 was provided as a gift from Dr. Catherine Labbaye (at the Istituto Superiore di Sanita, Rome, Italy). The null vector pGEM (Promega, Madison, WI) was used in place of the GATA-1 vector in separate wells, which served as the experimental control. A total of 24 experimental wells were used in this experiment ($n = 3$). The cells were exposed to conditions of low Ca (0.2 mM) or high Ca (3.2 mM), and likewise the absence or presence of PTH (50 nM). VDR-luciferase or pGEM constructs were transfected into the cells for 3 days before the changes in Ca and PTH were applied. The data were analyzed using a luminometer to measure the light produced from luciferase reacting with a luciferin substrate (Promega).

Statistical analysis

Results are expressed as means ± SE, and significance was determined by analysis with an unpaired student's *t* test (*) for two-group comparison or ANOVA for multiple group comparison analysis followed by the Tukey–Kramer analysis of multiple comparisons (†), and a *p* value less than 0.05 was considered significant.

Results

Diets and serum Ca

The serum Ca concentration in the rats fed a control, normal Ca (NC), normal phosphate, and adequate vitamin D_3 diet (Cntrl diet) was 10.5 mg/dl, and increased to 12.4 mg/dl in response to two sequential 1 μg doses of $1,25(OH)_2D_3$ treatment (hypercalcemia, HC), whereas the serum Ca concentration in Ca deficient rats given adequate vitamin D_3 was 8.0 mg/dl (hypocalcemia, LC). Serum Ca concentration in LC/DD rats was 6.5 mg/dl and rose to 9.5 mg/dl following two sequential 1 μg doses of $1,25(OH)_2D_3$ (LC/DD/$1,25D_3$).

Severe hypocalcemia blunts renal but not intestinal gene expression by $1,25(OH)_2D_3$

The effects of LC with vitamin D deficiency (LC/DD) and the same diet with $1,25(OH)_2D_3$ treatment (LC/DD/$1,25D_3$) were studied on transcriptional regulation of genes for renal cortex 1α- and 24-OHases and intestinal duodenum 24-OHase (Figs. 1A, C, and E). Renal cortex 1α-OHase transcript was significantly stimulated in the LC/DD diet, but treatment of $1,25(OH)_2D_3$ was not effective in repressing 1α-OHase gene expression in the LC status (Fig. 1A). In comparison, the LC/DD diet led to only a minimal decrease in renal cortex 24-OHase, but again, $1,25(OH)_2D_3$ treatment was not effective as an inducer of renal 24-OHase gene expression in the LC status (Fig. 1C), whereas, $1,25(OH)_2D_3$ treatment very potently induced intestinal duodenum 24-OHase gene expression (Fig. 1E).

LC and HC are better models of reciprocal renal hydroxylase regulation than classic models

Next, the effects of LC diet with adequate vitamin D_3 (LC) and NC diet with adequate vitamin D_3 plus $1,25(OH)_2D_3$ treatment (HC) were studied on transcriptional regulation of genes for renal cortex 1α- and

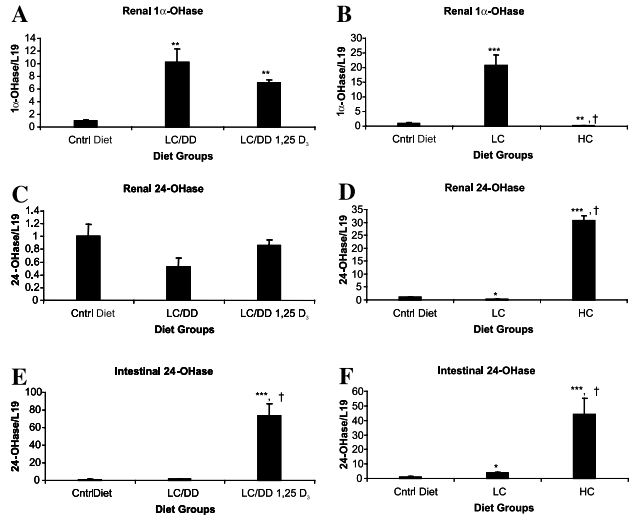


Fig. 1. Diet effects on renal and intestinal vitamin D hydroxylases assessed by real-time RT-PCR measurement: (A and B) renal 1α-OHase gene expression, (C and D) renal 24-OHase expression, and (E and F) intestinal 24-OHase gene expression. The control diet (Cntrl) consisted of normal calcium (0.47%), normal phosphate (0.3%), and an adequate vitamin D_3 diet. LC/DD refers to a low Ca (0.02%), normal phosphate (0.3%) diet, and deficient dietary vitamin D_3 (i.e., no vitamin D_3). Hypercalcemia (HC) was produced with a diet that consisted of normal dietary Ca (0.47%), normal phosphate (0.3%), and adequate vitamin D_3, plus $1,25(OH)_2D_3$ treatment. LC refers to a low Ca (0.02%), normal phosphate (0.3%) diet with adequate dietary vitamin D_3 levels. Adequate vitamin D_3 was administered orally at 4 μg per day for 20 days. In the cases of $1,25(OH)_2D_3$ treatment, rats were given two sequential 1 μg intraperitoneal injections of $1,25(OH)_2D_3$ (groups LD/DD and HC), at 16 and 4 h prior to sacrifice. Statistical analysis was done using the student's unpaired *t* test for independent comparisons between Cntrl diet and other diets, and Tukey–Kramer analysis of multiple comparisons when considering differences between LC/DD and LC/DD/1,25-D_3 ($^{\dagger}p < 0.001$), or LC and HC group comparisons ($^{\dagger}p < 0.001$). The relative values of hydroxylases to L19 were plotted (student's *t* test $^{**}p < 0.01$; $^{***}p < 0.001$) $n = 5$.

24-OHases and intestinal duodenum 24-OHase (Figs. 1B, D, and F). In this series of studies, renal cortex 1α-OHase was extremely up-regulated the LC condition, but this time $1,25(OH)_2D_3$ treatment (HC rats) significantly repressed renal cortex 1α-OHase (Fig. 1B). LC diet resulted in a significant repression of 24-OHase transcript in the renal cortex (Fig. 1D), and a significant increase of 24-OHase transcript in the duodenum (Fig. 1F). Treatment of $1,25(OH)_2D_3$ to the rats fed a normal Ca adequate vitamin D_3 diet (HC) resulted in greatly induced 24-hydroxylation gene expression in both the renal cortex (Fig. 1D) and duodenum (Fig. 1F). Unlike the classic deficiency models, both LC and HC represented models of strong reciprocal regulation of renal hydroxylases. The focus of the remainder of this study was specifically on differential gene expression patterns between the *LC* and *HC* groups, which showed the greatest degree of differential regulation of 1α-OHase and 24-OHase gene expression in the kidney cortex.

Microarray analysis: molecular determinants in renal cortical tissue

To ascertain the differential transcriptional regulation involved in PTH-mediated 1α-hydroxylation in the proximal kidney, highly purified RNA was made for microarray analysis from kidney cortex of rats fed one of two diets: (1) LC, deficient dietary Ca/normal vitamin D_3, which produced hypocalcemia, secondary hyperparathyroidism, high serum $1,25(OH)_2D_3$ concentrations, and repressed renal VDR, or (2) HC; normal dietary Ca/adequate vitamin D_3 plus bolus dosing of $1,25(OH)_2D_3$ by two consecutive treatments of 1 μg/dose, as previously described [24].

Excluding ESTs, control genes, and gene expression data of low signal intensity, this analysis revealed that 117 genes were 1.8-fold or greater differentially up-regulation by the hypocalcemic diet, LC, and 349 genes were differentially up-regulated by the hypercalcemic diet protocol, HC. The differential regulation of the calciotropic factors in the kidney cortex, Fig. 2, in LC versus HC conditions, demonstrated elevated gene expression for 1α-OHase (4.6-fold, Fig. 2A). In contrast, several other factors known to be regulated by $1,25(OH)_2D_3$ showed increased differential gene expression in the HC model, including 24-OHase (10.4-fold, Fig. 2B), calcium-binding protein-$_{28kDa}$ (6.2-fold, Fig. 2C) and osteopontin (2.6-fold, Fig. 2D). In addition, several fold differential regulation of VDR (4.0-fold, Fig. 2E) and vitamin D-binding protein (5.4-fold, Fig. 2E) was also observed in HC with respect to their values in LC.

Relative changes in top gene expressions in LC and HC conditions

Tables 2 and 3 depict a comparison of statistically significant differential gene expression patterns in kidney cortex by exposure to LC versus HC conditions. Table 2 shows fold increases in gene expression of genes regulated in LC, and the top 16 genes are listed with 1α-OHase (4.6-fold) as the seventh highest regulated gene in LC. Pax-6 (10.4-fold) an early developmental gene was the highest regulated gene in the LC subset, followed by GABA-B receptor 1d (6.6-fold), and the 9 kDa calcium-binding protein (5.6-fold). Table 3 depicts similar data to those in Table 2 but for the 20 most differentially regulated genes at the HC end of the array relative to LC showing 24-OHase (10.4-fold) as the fifth highest regulated gene in the HC set. γ-Glutamylcysteine synthetase (12.6-fold) a rate-limiting enzyme in synthesis of renal glutathione was the highest differentially regulated gene in the HC set, followed by UDP-glucuronosyltransferase (12.6-fold), and protein synthesis initiation factor 4AII and protein elongation factor (11.8-fold), respectively.

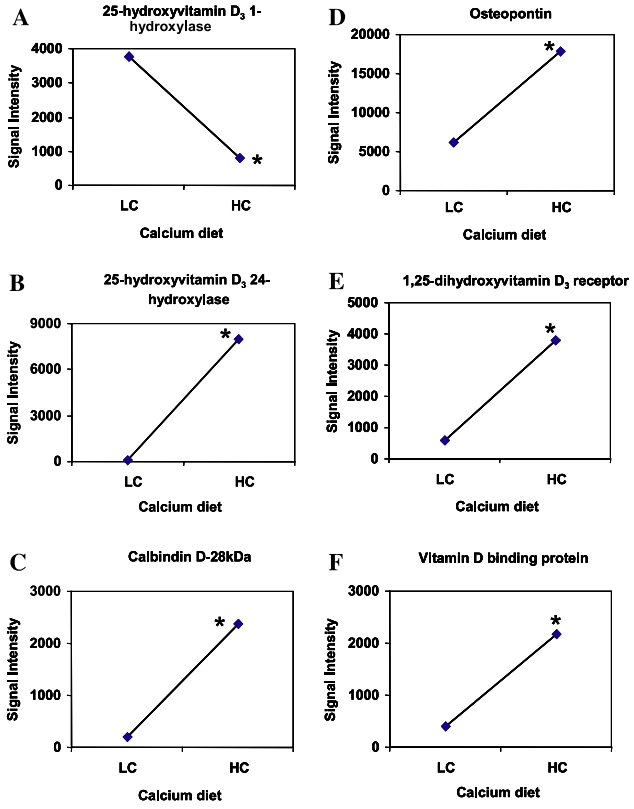


Fig. 2. Microarray results showing regulation of vitamin D system genes in hypocalmia (LC) versus hypercalcemia (HC), as defined in Fig. 1. Each plot represents the change in signal intensity of the specified genes in LC and HC models. (A) 25-Hydroxyvitamin D_3 1α-OHase decreased in the HC model relative to the LC model. In contrast, gene expressions showing increases in HC relative to the LC model were: (B) 25-hydroxyvitamin D_3 24-OHase, (C) calbindin D_{-28kDa}, (D) osteopontin, (E) $1,25(OH)_2D_3$ receptor (VDR), and (F) vitamin D-binding protein (DBP). The statistical analysis was accomplished using the Affymetrix Microarray Suite software package, where (*), equaled $p < 0.05$. In each case of differential gene expression between LC and HC statistical significance was met.

Gene expression changes in intracellular intermediates in LC and HC conditions

Table 4 shows signal intensities and differential fold changes of gene expression patterns for several signaling intermediates that were differentially elevated in either LC or HC. In LC, at least twofold increases were demonstrated for secreted phosphoprotein 24 (Spp2), cytosolic retinol-binding protein, CCAAT/enhancer-binding protein-β (C/EBPβ), cyclooxygenase-1, the protein-tyrosine phosphatase (SHP-1), and MAP kinase kinase (MKK2). The endopeptidase factor furin was 2.4-fold and the v-fos, transformation effector protein was 1.8-fold increased, respectively, in LC relative to the HC condition. However, in the HC environment there were numerous regulated elevations of signaling intermediates including calcium-independent phospholipase A2 (PLA2i), several forms of protein phosphatase, phosphoinositol 4-kinase (PI4K), protein kinase C-ζ interacting protein, RhoGAP, CaM kinase, p38 mitogen activated kinase, and phosphoinositide-specific phospholipase C form-I. Many of these are important in CaSR activation, including PLA2i, PI4K, and RhoGAP.

Table 5 shows several gene expression patterns for EST sequences that were found increased in the LC condition. Of greatest interest were the changes observed in expression of CREB-binding protein (CBP) interacting protein, C/EBPβ, and MAP kinase kinase (MKK2) as likely intermediates in PTH-mediated cAMP/PKA cell signaling. Also, there was a distinct pattern of evidence for cell stress in LC exhibited by increases in myosin light chain 3, stathman-1, and BAD. In the HC model (lower section, Table 5), there was a distinct pattern of increase in RTK signaling intermediates. Note that some of the intermediates were not considered statistically significant between LC and HC groups, these are indicated by the ¥ symbol.

Functional gene expression changes in LC and HC conditions

Table 6 shows signal intensities and significant differential fold changes for subsets of gene expression patterns grouped into five functional categories for comparison to other similar studies involving microarray in the kidney. These categories include genes for: (1) receptors, (2) transcription factors, (3) protein metabolism, (4) proteosomal enzymes, and (5) growth factors. Notable changes in the gene expression of receptors in HC occurred for growth hormone receptor, angiotensin II receptor, fibroblast growth factor receptor 1 (FGFR1), whereas PTHR1 was increased significantly in LC. Aside from histone H2a, CREB, and GATA-1, all of the other noted gene expression changes in the transcription factors were increased going from LC to HC conditions. Dramatic changes in signal intensity were observed for the protein initiation and protein elongation factors, as noted in Table 2. The condition of HC was conducive for increasing ubiquitin ligase (NEDD4) and several forms of ubiquitin conjugating enzyme, whereas, ubiquitin itself was highest in LC. Of interest was the 8.8-fold differential regulation of epidermal growth factor (EGF) gene expression in HC compared to LC conditions.

Real-time RT-PCR: confirmation of gene expression patterns in LC and HC

The results in Table 6 under the heading transcription factors demonstrate a 4.8-fold increase in the c-AMP response element-binding protein (CREB) and a similar 4.0-fold increase in the globin activating factor (GATA-1) in hypocalcemia, suggesting that these two genes may

Table 2
Top renal cortical gene regulations in hypocalcemia (LC) by fold difference in signal intensity

Accession #	Gene name	Fold difference
S74393	Pax-6	10.4
X60328	Cytosolic epoxide hydrolase	7.6
AB016161	GABAB receptor 1d	6.6
K00994	Intestinal calcium-binding protein	5.6
X05472	2.4 kb repeat DNA right terminal region	5.6
U19485	Secreted phosphoprotein 24 (Spp2)	5.0
AF000139	25-hydroxyvitamin D 1-hydroxylase	4.6
M20131	Cytochrome P450IIE1	4.6
U95113	Histone H2a	4.2
M19257	Cytosolic retinol-binding protein	4.0
M33648	3-OH-3-methylglutaryl-CoA synthase	4.0
L37333	Glucose-6-phosphatase	3.8
X63594	RL/IF-1	3.8
M94919	Beta-globin	3.6
X04979	Apolipoprotein E	3.6
S68245	Carbonic anhydrase IV	3.4

Table 3
Top renal cortical gene regulations in $1,25(OH)_2D_3$-hypercalcemia (HC) by fold difference in signal intensity

Accession #	Gene name	Fold difference
J05181	Gamma-glutamylcysteine synthetase	12.6
S56937	UDP-glucuronosyltransferase	12.6
U64705	Protein synthesis initiation factor 4AII	11.8
EST196604[a]	Protein elongation factor 2	11.8
L04619	25-hydroxyvitamin D 24-hydroxylase	10.4
M21476	Iodothyronine 5-monodeiodinase	10.4
AF048828	Voltage dependent anion channel	10.2
AFFX	Gene encoding cytoplasmic beta-actin	10.0
M74494	Sodium/potassium ATPase	9.8
U81186	17 beta-hydroxysteroid dehydrogenase	9.8
Z83757	Growth hormone receptor	9.8
M90065	Angiotensin II receptor	9.6
AA686031	NADH-ubiquinone oxidoreductase	9.2
X12748	Epidermal growth factor precursor	8.8
D88190	Protein kinase PASK	8.6
U17133	Zinc transporter ZnT-1	8.6
AF099093	Ubiquitin-conjugating enzyme UBC7	8.4
Y17295	1-Cys peroxiredoxin	8.4
S54008	Fibroblast growth factor receptor 1	8.2
U97146	Calcium-independent phospholipase A2	8.2

[a] Gene identified from the EST set of data.

be influenced by the condition of low Ca and elevated PTH concentration. This possibility was confirmed by using real-time RT-PCR to measure VDR, CaSR, CREB, and GATA-1 gene expression in the kidney cortex of the Cntrl diet group versus the LC group (Fig. 3). These results showed a decrease in VDR in LC ($p < 0.01$). There was no notable change in CaSR, however, significant increases in CREB and GATA-1 ($p < 0.01$, respectively) were detected in LC conditions (Figs. 3A–D).

We went on to test if GATA-1 has a specific role in regulating VDR in human proximal HKC-8 cells since its increase in LC correlated with VDR decrease and because several GATA-1 response elements have been demonstrated to exist on the VDR promoter. In Fig. 4, transient overexpression of GATA-1 into HKC-8 cells demonstrated a potent effect of GATA-1 to repress VDR constitutive expression in both low Ca and high Ca conditions. PTH also decreased VDR promoter activation in low Ca medium, however, the PTH-mediated repression of VDR promoter was abrogated in the presence of high Ca medium.

Changes in FGF receptors and sodium-dependent phosphate co-transporters

Fig. 5 shows both up- and down-regulations of fibroblast growth factor receptors (FGFR) and sodium-dependent phosphate co-transporters (NaPi II) depicted in plot format comparing directional changes in LC compared to HC. The data indicate a significant in-

Table 4
Regulation of cellular intermediates in hypocalcemia (LC) and hypercalcemia (HC)

Gene name	LC signal intensity	HC signal intensity	Fold differences
Intermediates increased in hypocalcemia			
Secreted phosphoprotein 24 (Spp2)	35992.6	8071.9	5.0
Cytosolic retinol-binding protein (CRBP)	14140.4	3136.0	4.0
C/EBP β	2466.6	985.1	3.2
Cyclooxygenase 1 (Cox-1)	3964.8	1476.4	3.2
Protein-tyrosine phosphatase (SHP-1)	4198.1	1479.4	3.0
Furin	6943.2	2447.7	2.4
MAP kinase kinase (MKK2)	10835.4	5497.1	2.2
v-fos, transformation effector protein	39709.2	21188.4	1.8
Intermediates increased in hypercalcemia			
Ca-independent phospholipase A2 (PLA2)	104.8	1613.3	8.2
Ras-related protein (Ras-rP)	150.2	3510.2	8.0
Protein Phosphatase 2A	194.6	2606.0	7.0
Casein kinase I α L	272.7	3487.2	6.2
Serine/Phosphatase protein kinase TAO1	207.0	2446.1	5.6
Protein Phosphatase 1 β	949.2	6774.7	5.4
Phosphatidylinositol 4-kinase	360.6	1828.1	5.4
PKC-zeta-interacting protein	1568.3	11713.1	5.4
Casein kinase 1 γ 3 isoform	212.8	1229.8	5.0
RAC protein kinase α	257.7	1707.9	4.8
Protein Phosphatase 2C	91.9	823.4	4.8
Guanylate cyclase-A	377.5	2272.8	4.8
Phosphatase 2A α	1497.2	7899.3	4.8
RhoGAP	199.5	1006.0	4.6
Ca/calmodulin-dependent protein kinase II δ	122.1	691.5	4.4
p38 mitogen activated protein kinase	182.4	1006.8	4.0
A-kinase anchor protein 121	461.8	2554.3	3.8
Tyrosine kinase receptor (Ptk-3)	603.3	1659.2	3.8
Diacylglycerol kinase	575.7	2280.4	3.8
Phosphoinositide-phospholipase C form-I	2468.8	7099.4	3.8
Protein tyrosine phosphatase	226.0	1567.2	3.6
Phospholipase D	556.1	1610.2	3.4
Protein kinase C δ	1353.6	2946.8	2.4
Protein Phosphatase 1 α	3904.8	10594.4	2.4
Protein Phosphatase 1 γ	4983.7	9350.8	2.4
A-kinase anchoring protein 220	722.9	1784.9	2.0
Ras-homologous GTPase	1466.7	3139.2	2.0

The data are displayed as both rat U34A Affymetrix GeneChip signal intensity changes between LC and HC groups, and as the fold difference representing the change in intensities between the two groups.

crease in FGFR1 (Fig. 5A) from a very low signal intensity in LC to >3000 as a signal intensity. There were no significant changes in FGFR2 or FGFR4, and FGFR3 was not represented in the array. The main co-transporter that accounts for as much as 70% of all renal proximal phosphate reabsorption, NaPi-2α, was significantly increased going from LC to HC (9.0-fold), and NaPi-2β and NaPi-2γ were significantly decreased going from LC to HC (2.0-fold and 2.4-fold, respectively) (Figs. 5D–F).

Discussion

In this study, GeneChip oligonucleotide microarray was utilized to exploit the differences in renal cortex gene expression between the two opposing conditions of hypocalcemic hyperparathyroidism versus $1,25(OH)_2D_3$-induced hypercalcemia, LC versus HC. Given the reciprocal nature of 1α-OHase and 24-OHase in the renal proximal tubule [25–29], the overall aim of our study was to examine the effects of polarized conditions of renal vitamin D metabolism to screen for factors most relevant to the renal proximal 1α-OHase and 24-OHase. In the HC model, $1,25(OH)_2D_3$ treatment resulted in acute but potent effects on $1,25(OH)_2D_3$ dependent gene expression (i.e., 24-OHase, Ca-binding protein-$_{28kDa}$ and osteopontin), and the attendant rise in blood Ca^{2+} concentration appeared to activate additional signals. The results indicate that the regulation of renal proximal vitamin D metabolism involves complex and coordinated effects of both extracellular ionized Ca^{2+} and PTH, and also involve fine tuned adjustments by $1,25(OH)_2D_3$ to a degree not previously appreciated.

This study clearly demonstrates a forceful decrease of VDR gene expression in the renal cortex of hypocalce-

Table 5
Signal intensities (LC and HC groups) and fold differences of relevant EST sequences on the rat U34A Affymetrix GeneChips

Gene name (Accession No.)	LC signal intensity	HC signal intensity	Fold differences
EST factors increased in hypocalcemia			
Potassium channel tetramerization domain, AA859966	339625.2	9379.1	10.0
Cyclin M4, AA859983	2905.9	890.3	4.6
CBP interacting protein, AA900476	8691.0	2976.2	3.4
Matrix Gla protein, AI012030	38641.7	12210.9	3.4
Myosin light chain 3, AA875523	10875.2	3214.2	3.4
Rho guanine nucleotide exchange factor, AA859562	5128.2	1545.9	3.2
Stathmin-1, AI231821	8611.0	3221.3	3.0
Apolipoprotein M, AA893213	33844.8	12330.3	3.0
NimA-related protein kinase, AI045249	1949.4	737.3	3.0
Mss4, PI4P 5-kinase, AI014135	39895.4	16456.4	2.8
Steroid receptor chaperone, tetratricopeptide, AA892378	29060.2	11524.8	2.8
MAP kinase kinase (MKK2), AA963674	26666.5	10651.0	2.4
Bcl2-associated death promoter (BAD), AA818072	7334.5	2467.0	2.4
GTP-binding signal transduction protein, AA875225	12761.9	5477.6	2.2
RTK related factors increased in hypercalcemia			
Signal transducing adaptor molecule[a], AI639410	104.8	1613.3	8.2
Ras-related protein, X12535	150.2	3510.2	8.0
PSD-95/SAP90-associated protein-1¥, U67137	128.0	1349.7	7.8
Glucose regulated protein, 9 kDa,	304.8	2974.6	6.8
RAC protein kinase alpha¥, D30040	257.7	1707.9	4.8
RAC protein kinase gamma¥, D49836	40.3	554.5	4.8
RAC protein kinase beta¥, D30041	218.8	1121.7	4.8
Extracellular signal-related kinase (ERK2)¥, M64300	346.6	1124.5	4.0
14-3-3protein, D30740	2987.1	8114.0	3.8
Tyrosine kinase receptor (Ptk-3), L26525	603.3	1659.2	3.8
Protein tyrosine Phosphatase, D38072	226.0	1567.2	3.6
SP120, D14048	3863.9	11562.3	2.4
Tim17, AB006450	2169.7	9825.0	2.2
Extracellular signal-related kinase (ERK3), M64301	1123.5	1909.7	1.6
Guanine nucleotide dissociation stimulator, L07925	1111.6	2151.8	1.8
Tim23¥, AB006451	8729.3	15120.4	1.8
Ras-related GTPase, X85183	1959	2111.2	1.2
Cdc42-binding kinase, AF021936	273.2	754.6	0.8
GSK-3beta interacting protein, AF017756	2319.2	3360.4	0.6
GEF-2¥, AB003515	8463.7	11638.6	0.2

This data set includes genes identified using the NCBI GenBank BLAST program which were present as ESTs.
[a] Gene identified from the EST set of data. Affymetrix values that were not considered statistically significant between LC and HC groups are indicated by the (¥) symbol.

mic rats and an inability of exogenous $1,25(OH)_2D_3$ to repress 1α-OHase or induce renal cortical 24-OHase gene expression in rats fed a LC diet (Figs. 1A, C, and E). In contrast, complete repression of renal 1α-OHase gene expression and maximal induction of renal and intestinal 24-OHase gene expression were demonstrated following treatment with $1,25(OH)_2D_3$ in normal Ca fed rats (Figs. 1B, D, and F). These opposing effects of $1,25(OH)_2D_3$ on renal vitamin D hydroxylases are suggestive of a role of extracellular Ca^{2+} in stabilizing VDR numbers to regulate 1α-OHase repression. A similar mechanism exists in the parathyroid gland where extracellular Ca regulates VDR and this is proposed to play a role in $1,25(OH)_2D_3$ repression of the PTH gene [30]. A possible role for Ca influenced regulation of VDR by $1,25(OH)_2D_3$ was demonstrated in the kidney [31]. The present study also supports the concept put forth by Iida et al. [19] that a link exists between proximal tubule decrease in VDR gene expression and increased 1α-OHase gene expression. Other studies portray VDR as non-essential in the mechanism of renal 1α-OHase repression because serum $1,25(OH)_2D_3$ concentration can be controlled by a diet high in vitamin D_3, Ca, and lactose when given to VDR null mice [32]. In vitro promoter studies, however, conclude that high extracellular Ca is at best a weak direct regulator of 1α-OHase gene repression [33,34], and the repression mechanism must therefore involve cross-talk among multiple autocrine/paracrine and endocrine factors to effect changes in gene transcription.

Microarray analysis of LC versus HC renal cortex mRNA gene expression revealed a number of differentially regulated genes with known and suspected calciotropic roles in kidney. CaSR was relatively unaffected by the two extremes, whereas PTHR1 was differentially increased (1.8-fold) in renal cortex exposed to LC. This

Table 6
Signal intensities(LC and HC groups) and fold differences depicted as categories of receptors, transcription factors, genes involved in protein synthesis, proteosomal enzymes, and potential growth factors

Gene name	LC[a] signal intensity	HC signal intensity	Fold difference
Receptors			
Growth hormone receptor	33.5	1030.4	9.8
Angiotensin II receptor	41.1	1608.2	9.6
Lactogen receptor	797.0	6630.6	6.0
Vitamin D receptor	582.5	3810.0	4.0
Retinoic acid receptor	5114.1	10892.6	2.0
Leptin receptor	669.9	1393.1	2.0
Vasopressin V2 receptor	3382.7	5664.7	2.0
Parathyroid Hormone receptor1	**33399.0**	17806.6	1.8
Transcription factor			
TFIIA	139.0	1116.3	6.4
CREB	**240.2**	33.0	4.8
CCAAT-binding transcription factor CBF[b]	194.9	1729.0	4.4
Histone H2a	**5330.3**	1157.9	4.2
GATA-1	**2099.2**	390.1	4.0
HES-1	627.0	2563.5	3.8
Rev-ErbA-alpha	1337.9	3829.3	3.0
UBF2	775.1	1404.2	3.0
Rev-ErbA-beta	808.3	2140.3	2.8
POU domain factor (Brn-5)	75.8	656.8	2.6
Hepatocyte nuclear factor 4	2064.7	6987.0	2.4
Pax-8	2094.7	4555.2	2.2
Cyclin D1	3668.2	11537.3	1.8
Protein synthesis			
Protein synthesis initiation factor	1924.8	13738.8	11.8
Protein elongation factor-2[b]	169.9	9087.4	5.9
Proteosomal enzymes			
Ubiquitin conjugating enzyme Ubc7	107.6	1437.2	8.4
Ubiquitin ligase (Nedd4) protein	148.9	3012.2	7.8
Ubiquitin conjugating enzyme Ubce2a	243.5	1874.9	6.8
Ubiquitin conjugating enzyme Ubc2e	332.1	1236.1	4.2
Proteasome subunit RC8	692.9	2994.8	3.6
Ubiquitin conjugating enzyme Ubc4a	988.8	2161.7	2.4
Ubiquitin	**66466.6**	27737.6	2.4
Growth factors			
Epidermal growth factor precursor	819.7	21409.3	8.8
Fibroblast growth factor 9	1425.6	2440.0	8.2
Insulin-like growth factor I	29.8	393.7	7.6
Vascular endothelial cell growth factor	92.0	878.0	6.4
Insulin-like growth factor-binding protein-2	263.5	1798.6	3.8
Insulin-like growth factor-binding protein-5	685.5	1992.0	3.6
Bone morphogenetic protein 4	1382.3	2041.2	2.4
Leptin	670.0	1393.0	2.0

[a] Bold denotes increased gene expression in LC.
[b] Genes identified from the EST set of data.

latter effect is in contrast to PTHR1 regulation studied in whole kidney [35], but may relate to an enhanced activity of PTH in the proximal kidney during chronic LC conditions. Intracellular vitamin D-binding protein (iDBP), VDR, calbindin-$_{28kDa}$, osteopontin, and 24-OHase were all low in hypocalcemic conditions and elevated in hypercalcemic conditions, which is consistent with other reported literature on these factors [36,37]. The degree to which the repression of calbindin-$_{28kDa}$ and osteopontin in the LC condition relates to VDR loss is unclear, but both molecules are known to be increased in response to $1,25(OH)_2D_3$ when serum Ca concentrations are at least normal [18,38]. The high expression of secreted phosphoprotein-24 (Spp2) in LC was an intriguing finding since this factor was reported to be associated with fetuin mineral complex in serum and play a role in mineralization of bone [39]. Spp2 has an N-terminal cystatin domain, which predicts an involvement of Spp2 to inhibit activity of thiol proteases, like cathepsins and caspases [39,40]. The regulation of Spp2 was mirrored by a similar elevation in matrix gla-protein (EST; AI012030).

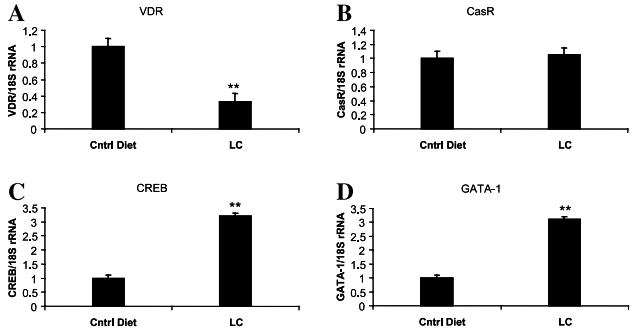
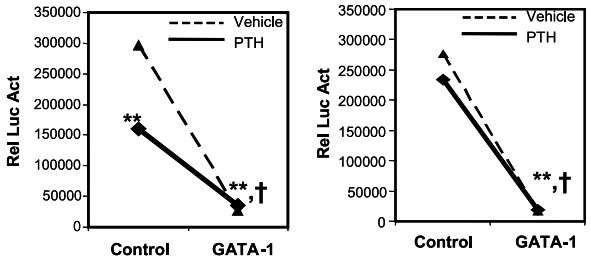


Fig. 3. Real-time RT-PCR of cortical kidney samples from the control normal calcium (Cntrl diet) and LC diet groups previously described. Cntrl diet is the same as HC, but without the sequential doses of $1,25(OH)_2D_3$. Each result confirms the previous data discovered in microarray. Each gene was analyzed by its own specific TaqMan probe and normalized to the 18s rRNA housekeeping gene. Statistical analysis was done using the student's unpaired t test ($^{**}p < 0.01$), $n = 3$.



(A) HKC-8 low calcium (LC) VDR promoter.

(B) HKC-8 high calcium (HC) VDR promoter.

Fig. 4. Overexpression of GATA-1 protein into renal proximal HKC-8 cells, and the effect of PTH and GATA-1 on VDR gene expression. GATA-1 transient transfections were tested in the presence of HKC-8 cells cultured in either, low, 0.2 mM, or high, 3.2 mM, Ca in the medium. The effect of PTH to decrease VDR promoter activity was dependent upon Ca, whereas, the effect of GATA-1 to repress VDR promoter was independent of Ca. Data are expressed as relative luciferase activity. Statistical analysis was done using the student's unpaired t test (**,†; $p < 0.01$), $n = 3$.

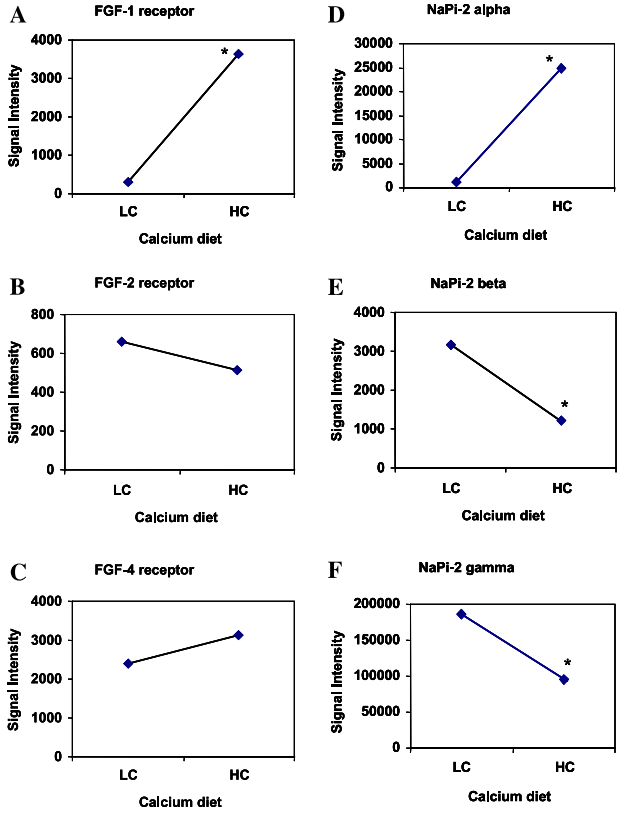


Fig. 5. Microarray results showing both up- and down-regulation of FGF receptors and NaPi II co-transporters in hypocalcemia (LC) versus hypercalcemia (HC). The graph represents a significant increase in: (A) FGF-1 receptor, but significant differential expression of genes for (B) FGF-2 and (C) FGF-4 receptors. The (D) NaPi-2α showed a significant increase from LC to HC and (E) NaPi-2β and (F) NaPi-2γ showed significant decreases. NaPi-2α was determined after Blast analysis of the EST195529, Accession No. AA891726. Therefore, NaPi-2α does not appear in Table 2. The statistical analysis was accomplished using the Affymetrix Microarray Suite software package, where (*), equaled $p < 0.05$.

As expected, 1α-OHase gene activation was increased in the hypocalcemic condition as opposed to a much lower level in the hypercalcemic condition in the microarray. These results of reciprocal regulation of 1α- and 24-OHases served to validate the use of microarray to explore the conditional and differential regulation of gene sets related to either LC or HC extremes [41], and correlate these gene expression patterns with changes in either renal proximal VDR or 1α-OHase gene expression [42]. As an example, this study identified two noteworthy transcription factors, CREB and GATA-1. Both transcription factors were confirmed to be differentially expressed in the LC condition by quantitative RT-PCR, and their signal intensities in the microarray analysis corresponded in magnitude with the level of expression of 1α-OHase in the LC condition, as well as the magnitude of expression with which VDR expression occurred in the HC condition. These transcriptional data are a limitation without functional protein data, however, initial experiments with GATA-1 transient overexpression in proximal kidney cells demonstrated a forceful decrease of VDR promoter activation suggesting that GATA-1, or its family members [43], can potentially regulate VDR in a tissue-specific manner. Further experimentation will be necessary to confirm this finding. However, computational analysis of the two promoters for 1α-OHase and VDR, respectively, using the TRANSearch program revealed multiple sites for CREB [44,45] and GATA-1 [45,46].

The phosphaturic action of PTH is well established in the proximal tubule, and this action best correlates with a PTH-mediated reduction in NaPi-2α cotransporters in the microvilli at the apical BBM [47], with no direct regulation in response to changes in serum phosphate [48]. Our study is supportive of these past findings. The mechanism

responsible for NaPi-2α down-regulation by PTH is believed to be an irreversible internalization of type II cotransporters [49]. Parathroidectomized (PTX) rats display a recovery of the NaPi-2α cotransporter protein that does not parallel the mRNA transcript [50], but our results would argue that transcript levels are down-regulated in the presence of high PTH and increase as a result of high Ca or by $1,25(OH)_2D_3$ treatment.

Having an adequate to high serum calcium concentration seems to favor involvement of RTK gene expression that would likely be triggered via EGF/FGFR pathway activation. FGF-23 is a novel phosphaturic factor that was recently shown in a transgenic model to repress 1α-OHase, leading to low serum $1,25(OH)_2D_3$ [51]. The actual kidney receptor for FGF-23 is not known, but the present results demonstrated that FGFR1 was markedly up-regulated in cortical kidney exposed to hypercalcemia [52–54]. High expression of FGFR1 at the HC end of the microarray suggests it may be a potential receptor for FGF-23 to modulate 1α-OHase repression. Alternatively, FGFR3 has been proposed to act as a receptor for FGF-23 in regard to renal phosphate handling [55]. The high (11.8-fold) differential increase of signal transducing adaptor molecule-2 (STAM-2), which contains a Src homology 3 (SH3) domain for tyrosine kinase growth factor receptor phosphorylation akin to growth factor receptor-protein bound 2 (Grb2) [56], is evidence of RTK-mediated activity within the acute time frame of the $1,25(OH)_2D_3$ treatment in HC.

Aside from the importance of acute $1,25(OH)_2D_3$ in calcium homeostasis, this study was also useful in directing attention to potential new aspects of $1,25(OH)_2D_3$ action in kidney. Increases in proteosome enzymes, growth factors, peptide receptors, transcription factors, and protein synthesis factors in the HC condition are indicative of very diverse and specialized processes potentially under Ca or $1,25(OH)_2D_3$ control. Particularly notable were the dramatic increases in EGF and angiotensin II receptor. EGF is a potent mitogen in many cell systems and has the potential to regulate VDR [57]. Activation of the angiotensin II receptor promotes EGF induced growth of renal proximal epithelium, and there also is strong evidence for the involvement of vitamin D in regulation of blood pressure, electrolyte balance, and fluid volume by functioning as a potent negative endocrine regulator of renin gene expression [58].

The LC and HC models used in this study identified potential growth factors, transcription factors, and receptors involved in the reciprocal regulation of the 1α-OHase and 24-OHase. Inverse regulation of the two vitamin D hydroxylases was achieved in the LC and HC models and low 24-OHase expression in LC was accompanied by a similar down-regulation of the VDR. In the LC model, up-regulation of 1α-OHase is PTH dependent. Whereas, in the HC model, 24-OHase up-regulation is due to $1,25(OH)_2D_3$ treatment but many genes that were up-regulated in HC do not necessarily contain a functional VDRE in their promoter. Since our HC model was produced by acute $1,25(OH)_2D_3$ treatment with attendant rise in serum Ca, up-regulation of renal genes could therefore be due to three possible mechanisms. First is increased gene expression induced by genomic effects of $1,25(OH)_2D_3$ leading to activation of genes directly by VDR binding on promoter VDREs. Second is gene expression regulation responding to non-genomic effects of the $1,25(OH)_2D_3$ pathway with coordinated involvement of RTK activation. Third is gene activation induced in response to increases in extracellular Ca^{2+} concentration and activation of the CaSR leading to signaling through MAP kinase pathway? The exact involvement of each of these factors and activation of different pathways leading to differential regulation of the hydroxylases would further have to be characterized in a kidney proximal convoluted tubule cell line model.

In summary, this study is the first to profile gene sets involved in the renal cortex in response to conditional exposure to chronic LC versus HC induced by acute $1,25(OH)_2D_3$ treatment. The design of the study exploited as much as possible the determinants involved in differential regulation of VDR and the two key proximal tubule vitamin D hydroxylases by conducting experiments with kidney cortex. Our data further suggest that the molecular regulation of 1α-OHase repression is complex, tissue-specific, and suggestive of a mechanism that coordinates adequate or high dietary Ca with stable gene expression of VDR, and that likely involves the influence of an independent factor, such as Ca^{2+} or FGF-23, that are counter-regulatory to the ac-

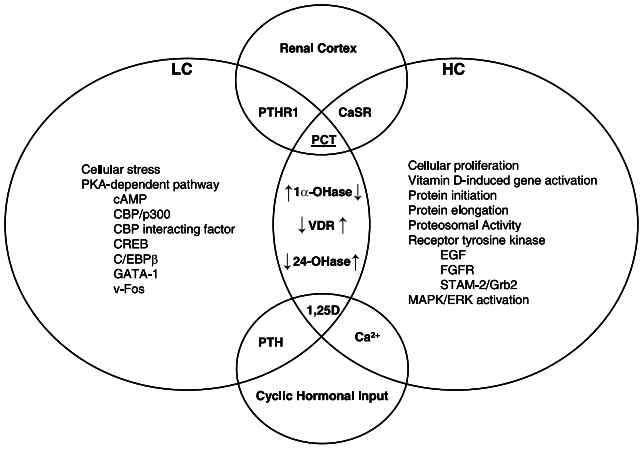


Fig. 6. A complexity model of gene expression in the proximal kidney in the transition from low to high calcium status. This model will serve to help make predictions as to the determinants regulating renal vitamin D metabolism as dictated by specific physiological changes. LC refers to low serum calcium, HC refers to high serum calcium, and PCT refers to proximal convoluted tubule. All other gene names are explained in the text above.

tion of PTH to increase 1α-OHase and decrease 24-OHase. Future experiments for the study of renal vitamin D metabolism should focus on the integrated, as well as independent effects of Ca^{2+}, PTH and $1,25(OH)_2D_3$ within defined units of the nephron, and in renal proximal versus distal cell lines. This approach would enable the field to better appreciate the specialized role of epithelial cells of the proximal tubule as the main endocrine cells of the vitamin D system.

Fig. 6 illustrates an integrative model based on the findings in this study and support the hypothesis that the cAMP/PKA pathway is a distinctive feature of LC in response to elevated PTH. As VDR in the PCT decreases in LC, proximal cells become refractory to elevated $1,25(OH)_2D_3$ synthesis. In HC conditions, $1,25(OH)_2D_3$ responsive gene activation/repression is intact. The model also predicts a role of CaSR activation that opposes PTH and a concerted involvement of RTK activation for ultimate control of renal proximal 1α-hydroxylation and phosphate transport.

Acknowledgments

We thank Darrel Hoy for assistance with animal handling, National Animal Disease Center (Ames, IA). We also thank Elisabeth A. Beckman for help with data processing and manuscript preparation.

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